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DOI: https://doi.org/10.2310/7290.2007.00015

RESEARCH ARTICLE

Multitracer Positron Emission Tomographic Imaging of Exogenous Gene Expression Mediated by a Universal Herpes Simplex Virus 1 Amplicon Vector

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Abstract

To develop efficient and safe gene therapy approaches, the herpes simplex virus type 1 thymidine kinase gene (HSV-1-*tk*) has been shown to function as a marker gene for the direct noninvasive in vivo localization of thymidine kinase (TK) expression by positron emission tomography (PET) using radiolabeled nucleoside analogues as specific TK substrates. Moreover, the gene encoding dopamine type 2 receptor (*d2r*) could be used as a PET marker gene using specific radiolabeled receptor binding compounds. Here we describe the quantitative colocalization of *d2r* and HSV-1-*tk* gene expression mediated from a universal HSV-1 amplicon vector in a subcutaneous human Gli36dEGFR glioma model by PET. The HSV-1 amplicon vector was constructed using a bicistronic gene cassette to contain (1) the *d2r80A* mutant, which is able to bind its ligand racloprid but unable to activate downstream signal transduction pathways, and (2) the *tk39* mutant with enhanced enzymatic activity toward guanosine analogues fused to the green fluorescent protein gene (*tk39gfp*) serving as a marker gene in cell culture. After infection of human Gli36dEGFR glioma cells with the HSV-*d2r80A*IRES*tk39gfp* (HSV-DITG) amplicon vector in cell culture, D2 receptor expression and its targeting to the cell surface were determined by Western blotting and immunolabeling. Vector application in vivo served for quantitative colocalization of *d2r80A*- and *tk39gfp*-derived PET signals employing the specific D2 receptor binding compound [^{11}C]racloprid and the specific TK39 substrate 9-(4-[^{18}F]fluoro-3-hydroxymethylbutyl)guanine. Our results demonstrate that for the range of gene expression studied in vivo, both enzymatic and receptor binding assays give comparable quantitative information on the level of vector-mediated gene expression in vivo. The *d2r80A* in combination with a specific binding compound passing the intact blood-brain barrier might be an alternative marker gene for the noninvasive assessment of vector-mediated gene expression in the brain using PET.

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This work was supported in part by the Deutsche Forschungsgemeinschaft (DFG-Ja98/1-2), Ministerium für Schule, Wissenschaft und Forschung NRW (MSWF 516-40000299), Center for Molecular Medicine Cologne (CMMC-TV46), 6th frame work European Union grant European Molecular Imaging Laboratories (LSHC-CT-2004-503569), and Diagnostic Molecular Imaging (LSHB-CT-2005-512146).

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DOI 10.2310/7290.2007.00015

GLIOMAS are the most common types of brain tumors, which invariably lead to death over months or years. The main diagnostic procedures in patients with suspected glioma combine modern histologic and genetic evaluations with improved imaging technologies based on positron emission tomography (PET) and magnetic resonance imaging (MRI).[1] The ultimate goal of molecular imaging by PET is the noninvasive localization and quantification of disease-specific molecular events in vivo, including endogenous or exogenous gene expression, signal transduction, protein-protein interaction, or transcriptional regulation.[2]

Gene therapy of gliomas is a promising treatment approach, although positive effects of gene therapy vectors in humans have been observed only in single patients bearing small tumors.[3–8] Localized transduction of brain tumor cells with therapeutic genes may influence their

biologic properties by rendering them sensitive to prodrugs, by altering the gene expression of cell cycle–regulating proteins, by inhibiting angiogenesis, by stimulating the immune response, or by virus vector–mediated oncolysis employing replication-conditional vectors.[2,9]

One of the most important issues for making gene therapy applicable to humans is the development of technologies for the noninvasive monitoring of the location, magnitude, and duration of vector-mediated gene expression in vivo.[10–12] Two radionuclide-based reporter gene approaches have been used: enzyme-based reporter genes whose products convert radionuclide-labeled specific substrates to trapped products and receptor-based reporter genes whose products bind radionuclide-labeled ligands. The herpes simplex virus type 1 thymidine kinase gene (HSV-1-*tk*) has been the most widely exploited enzyme-based reporter gene whose expression is detected by radionuclide product sequestration.[10,12] HSV-1-TK can phosphorylate a number of compounds that cannot be phosphorylated by endogenous mammalian thymidine kinase (TK) enzymes. The retention of the HSV-1-TK-dependent product in cells and tissues can be monitored by PET employing acycloguanosine substrates[13] and thymidine analogues.[14,15] The location and magnitude of HSV-1-TK expression could be followed by PET after retroviral,[14,16,17] adenoviral,[10,18,19] and herpes viral[20,21] vector–mediated HSV-1-*tk* gene transfer in animal models and after liposomal vector–mediated HSV-1-*tk* gene transfer in patients.[11] However, current HSV-1-TK substrates do not pass the intact blood-brain barrier.[11,22–24] Therefore, improved lipophilic HSV-1-TK substrates have to be engineered[25] or alternative marker gene/marker substrate combinations have to be developed that could be potentially applied in the brain.[26]

Two receptor-based reporter genes have been described that use radionuclide detection with potential application in the brain. The somatostatin type 2 receptor (SSTr2) is predominantly expressed in the pituitary. When used as a reporter gene, the location of SSTr2 expression can be monitored by systemic injection of a technetium 99m–labeled SSTr2 peptide ligand ([^{99m}Tc]-P829) and subsequent imaging by planar imaging techniques with a conventional gamma camera.[27] The dopamine type 2 receptor (D2R) is primarily expressed in the striatum and pituitary glands. This protein has seven transmembrane domains and a molecular weight of 105 kDa.[28] D2R has been used as a PET reporter gene in an adenoviral delivery vector and in stably transfected tumor cell xenografts, where the location, magnitude, and duration of D2R reporter gene expression was monitored by microPET using D2R-dependent binding of systemically injected 3-(2′-[^{18}F]-fluoroethyl)-spiperone (FESP) as a high-affinity positron-labeled D2R ligand.[29] One potential problem with receptor-based PET reporter genes is the possibility that ectopic receptor gene expression could change the target cell physiology as circulating endogenous ligands for the ectopically expressed receptors might constitute a chronic stimulus provoking undesired biologic responses. The primary response evoked by dopamine binding to the D2R is the activation of a G_i-protein complex that leads to inactivation of adenylyl cyclase and a decrease in cellular cyclic adenosine monophosphate levels.[30] Two mutations (D2R D80A and S194A) have been described that uncouple ligand binding of the D2R from activation of the $G_{\alpha i}$ subunit of the G_i-protein complex signaling pathway.[31–33] The usability of these mutated D2R genes to serve as PET marker genes has been demonstrated by Liang and colleagues.[34]

This study was designed to directly compare vector-mediated receptor (*d2r80A*) and enzyme (HSV-1-*tk*) expression mediated from a single bicistronic HSV-1 amplicon vector with respect to localization and quantification of gene expression. We demonstrate the utility of D2R80A and its blood-brain barrier–permeable ligand [^{11}C]racloprid to be used as a marker gene/marker receptor binding combination to image vector-mediated gene expression in a subcutaneous glioma model in nude mice. Most importantly, *d2r80A* and HSV-1-*tk* gene expression could be colocalized by microPET on a quantitative level.

Material and Methods

Cell Culture

Human Gli36dEGFR glioma cells (kind gift of Dr. David Louis, Molecular Neurooncology Laboratory, Massachusetts General Hospital, Boston, MA) were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS, Roche Diagnostics, Mannheim, Germany) and 100 U/mL penicillin and 100 μg/mL streptomycin (P/S, Life Technologies) at 37°C in a 5% CO_2/95% air atmosphere. Human Gli36dEGFR glioma cells were used in this study as their infectability by HSV-1 amplicon vectors has been shown to be very high.[35] Gli36dEGFR cells carrying a mutated epidermal growth factor receptor (EGFR) grow rapidly in vivo in comparison with wild-type Gli36 cells.

HSV-1 Amplicon Plasmid Construction

We generated an internal ribosome entry site (IRES)-based bicistronic coexpression vector carrying a rat D2R gene (*d2r80A*) and HSV-1-*tk39* as PET reporter genes with the *tk39* gene fused to the green fluorescent protein (GFP) gene (*tk39gfp*[21,36]). For this purpose the *d2r80A* gene (kindly provided by Dr. Gambhir, Crump Institute for Biological Imaging, Los Angeles and University of California-Los Angeles, CA) was amplified by polymerase chain reaction (PCR) using primers 5′-CAT TGC GCT AGC ATG GAC CCA CTG AAC CTG-3′ and 5′-CAT TGG GAT CCT CAG CAG TGC AAG ATC TTC AT-3′ (MWG Biotech, Ebersberg, Germany). The rat *d2r80A* template encodes a mutated D2R leading to uncoupling of the signal transduction pathway after binding of a ligand.[34] The PCR product was digested with *Nhe*I and *BamH*I and ligated into the plasmid pCD-mIRES-TK39(gly5)EGFP.[21] The *d2r80A* fragment and the first part of the IRES element (nucleotides [nt] 1–553 of the used IRES sequence) were subsequently excised using the restriction endonucleases *Nhe*I and *Hind*III and ligated into the plasmid pHSV-CD-IRES-TK39(gly5)-EGFP, leading to the plasmid pHSV-D2R80A-IRES-TK39(gly5)-EGFP (pHSV-DITG; Figure 1A). The two genes of interest (*d2r80A* and *tk39gfp*) are under the control of a common upstream cytomegalovirus (CMV) promoter and transcribed as a single transcript. Translation of the two genes was uncoupled with translation initiation of the upstream gene (*d2r80A*) being mediated by a cap-dependent mechanism while the translation initiation of the downstream gene (*tk39gfp*) occurs via the IRES. IRES functions as a ribosome-landing pad to facilitate efficient internal initiation of translation.

Helper Virus–Free Packaging of HSV-1 Amplicons

Helper virus–free stocks of the HSV-1 amplicon construct pHSV-DITG were generated by transient cotransfection of amplicon deoxyribonucleic acid (DNA) with a cosmid-set spanning the HSV-1 genome,[37] which had been mutated in *pac* sequences to inhibit packaging of the HSV-1 genome[38] as previously described.[21] Briefly, 10 cm plates were seeded with 3.6×10^6 vero 2-2 cells. After 16 hours, the cells were transfected with 6 μg of *PacI* digested cosmids and 1.8 μg of the HSV-1 amplicon plasmid using lipofectamine (Life Technologies) for 5.5 hours. Thereafter, cells were washed three times with serum-free medium and cultivated in packaging medium (DMEM, 6% FBS, 25 mM HEPES [pH 7.3]) at 37°C for 24 hours. Subsequently, the medium was replaced by fresh packaging medium and cells were cultivated for 24 hours at 34°C followed by cultivation at 37°C for 24 hours. After harvesting, vector stocks were purified and concentrated. In brief, harvested cells were centrifuged at 2,000*g* for 10 minutes and the pellet was resuspended in serum-free DMEM. To release HSV-1 virions, cells were frozen three times in a mixture of dry ice and ethanol, thawed in a 37°C waterbath, and subsequently sonicated at 75 W for 20 seconds. Cell debris was pelleted at 2,000*g* for 10 minutes, and the supernatant was filtered (0.45 μm). Thereafter, virus was pelleted by centrifugation at 20,000*g* at 4°C for 1 hour. The virus pellet was resuspended in phosphate-buffered saline (PBS), and virus stocks were stored at −80°C for titration and final use. Purified vector stocks were titrated (transducing units (tu) per milliliter; tu/mL) on Gli36dEGFR cells by infecting confluent monolayers in 24-well plates (Falcon, Becton Dickinson, Heidelberg, Germany) and subsequent counting GFP-expressing cells 24 hours later.

Functional Evaluation of D2R80A Receptor Expression

To analyze the expression of the mutated D2R, human Gli36dEGFR glioma cells were infected with HSV-DITG using multiplicities of infection (MOI) of 1 and 3. After 24 hours, cells were lysed in buffer containing 10 mM Tris pH 8.0, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 0.5% Triton X-100, and protease inhibitor mix (Complete mini, Roche Diagnostics GmbH, Mannheim, Germany), and the amount of protein present in each sample was quantified using the BCA Protein Assay (Pierce, Rockford, IL). Equal amounts of denatured protein were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to a polyvinylidene fluoride (PVDF) membrane (Amersham Pharmacia Biotech, Freiburg, Germany) in standard Tris-glycin transfer buffer. After blocking of nonspecific binding sites with 5% nonfat dry milk in 0.3% PBS-Tween 20 (PBST) for 1 hour, the membrane was washed three times with PBST and incubated for 1 hour with antibody sc-9113 recognizing the D2R (1:1,000 in 5% dry milk/PBST; Santa Cruz Biotechnology, Santa Cruz, CA). Afterward, the membrane was washed and incubated for 1 hour with the secondary antibody (1:10,000 in 5% dry milk/PBST; goat antirabbit Dianova). Proteins were visualized using the SuperSignal West Pico Chemiluminescence substrate (Pierce).

To analyze the appropriate localization of D2R80A into the plasma membrane, Gli36dEGFR cells were grown on

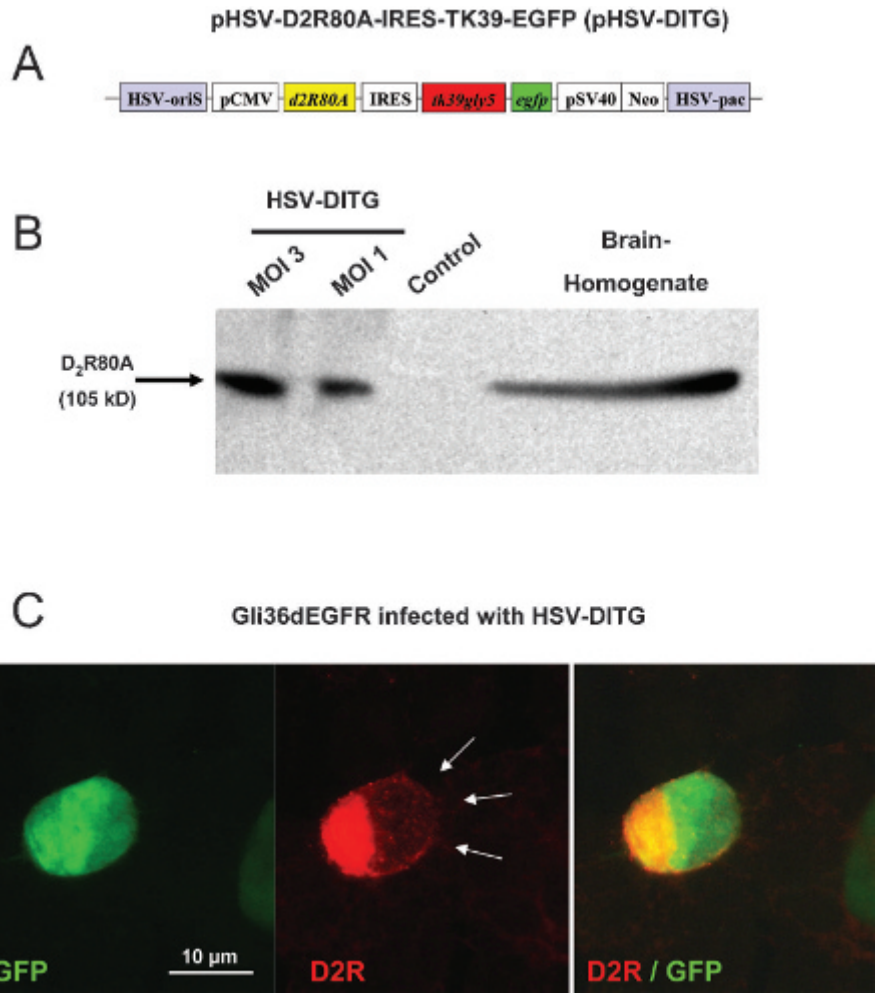

Figure 1. *A*, Structure of the herpes simplex virus 1 (HSV-1) amplicon vector. The sequence of the rat *d2r80a* gene was amplified by polymerase chain reaction and cloned into the HSV-1 amplicon. A 5-glycine linker is located between the open reading frames of *tk39* and *gfp*. *d2r80a* and *tk39gfp* genes are linked by an internal ribosome entry site (IRES). This HSV-1 amplicon plasmid carries two sequences derived from the HSV-1 genome, which are the HSV-1 *oriS* sequence serving as the site of origin of deoxyribonucleic acid (DNA) replication and the packaging signal HSV-1 *pac*, which mediates packaging of HSV-1 amplicon plasmid DNA into highly infectious HSV-1 particles. In addition, the HSV-1 amplicon carries a neomycin resistance gene (*Neo*) under control of the SV40 promoter (*pSV40*), an ampicillin resistance gene and an origin of DNA replication for propagation in *Escherichia coli*. *B*, Western blot analysis of HSV-1 amplicon vector–mediated D2 receptor (D2R) expression. Gli36dEGFR cells were grown to confluence and infected with HSV-DITG at multiplicities of infection (MOI) of 1 and 3. Negative control cells were left uninfected, and brain homogenate served as a positive control. Twenty-four hours after infection, cells were lysed and equal amounts of whole protein were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting using antibody sc-9113 recognizing the N-terminal region of the D2R. *C*, Localization of the mutated D2R. Gli36dEGFR cells were grown on coverslips and infected with HSV-DITG at MOI = 1. Twenty-four hours after infection, immunostaining using antibody sc-9113 recognizing the N-terminal region of the D2R was performed. *Arrows* indicate the localization of the D2R in the plasma membrane.

coverslips, infected with HSV-DITG (MOI 1), and immunostaining using antibody sc-9113 recognizing the D2R (Santa Cruz Biotechnology) was performed as follows: cells were washed three times with PBS and fixed in 4% paraformaldehyde/PBS for 20 minutes. After three 6-minute washes with PBS, cells were permeabilized with 0.2% Triton X-100/PBS for 3 minutes. Afterward, they were washed three times for 5 minutes and blocked 1 hour at 37°C with 3% bovine serum albumin (BSA/PBS) and incubated with the primary antibody (1:100 in 0.3% BSA/PBS) for 1 hour at 37°C. After three 5-minute washes with 0.3% BSA/PBS, cells were incubated for 1 hour with the secondary Cy3-conjugated antibody (FluoroLinkTM CyTM3-labeled goat antirabbit IgG, Amersham, Freiburg, Germany). The coverslips were rinsed three times with PBS and once with water and mounted in antifade reagent. Cells were analyzed using a Laser Scanning Microscope (Leica TCS SP II, Leica, Bensheim, Germany).

Generation of D2R80A-IRES-TK39(gly5)-EGFP Expressing Positive Control Human Gli36dEGFR Glioma Cells

To generate cell lines to be used as positive control tumors stably expressing D2R80A-IRES-TK39(gly5)-EGFP, 1×10^6 human Gli36dEGFR glioma cells were transfected with 1 μg pHSV-DITG using 48 μg lipofectamine (Invitrogen, Carlsbad, CA) in Optimem (Life Technologies, Carlsbad, CA). After incubation for 5 hours at 37°C, cells were washed with Optimem and cultivated in DMEM containing 10% FCS, 1% P/S, and 1,000 μg/mL G418 for selection. To generate tumor clones with various levels of GFP expression, the transfected cell population was sorted by means of fluorescence-activated cell sorting on a FACStar+ instrument (Becton Dickinson). Thereby, cells with low and high levels of TK39GFP expression were generated and designated Gli36dEGFR-DITG-R2 and Gli36dEGFR-DITG-R5, respectively.

Animal Experiments

All animal procedures were in accordance with the German Laws for Animal Protection and were approved by the local animal care committee and the Bezirksregierung Köln.

Tumor Model and Vector Application In Vivo

Wild-type human Gli36dEGFR glioma cells (10^6 cells in 100 μL of DMEM) were injected subcutaneously into both flanks of nude mice (≈30 g; $N = 12$). Injection of Gli36dEGFR-DITG cells stably expressing D2R80A-IRES-TK39(gly5)-EGFP in the neck served for the generation of positive control tumors ($n = 6$ with Gli36dEGFR-DITG-R2; $n = 6$ with Gli36dEGFR-DITG-R5). Three sets (four animals each) were studied ($N = 12$). After tumors had grown to a size of approximately 0.5 to 1 cm^3, the HSV-DITG amplicon vector (vector doses ranging between 1.8×10^7 and 2.4×10^8 tu in 200 μL of PBS) was injected into one of the subcutaneous growing Gli36dEGFR wild-type tumors by direct inoculation with the needle tract following the craniocaudal axis of the animal. To determine the primary transduction efficiency of HSV-1 amplicon vectors, PET was performed 24 hours after vector administration.

Radiosynthesis of 9-(4-[^{18}F]Fluoro-3-Hydroxymethylbutyl)Guanine

No-carrier-added [^{18}F]fluoride was produced by irradiation of 96% enriched [^{18}O]water (Rotem Industries, Beer Sheva, Israel) with 17 MeV protons via the $^{18}O(p,n)^{18}F$ reaction using an MC 17 cyclotron (Scanditronix, Uppsala, Sweden). The irradiated sample was delivered to the synthesis apparatus and the [^{18}F]fluoride trapped on a Chromafix 30 PS-HCO3 anion exchange cartridge (Macherey-Nagel GmbH, Düren, Germany). After the [^{18}O]water was recovered in a collection vial for recycling, the [^{18}F]fluoride was desorbed with 300 μL of a 70 mM aqueous K_2CO_3 solution and transferred to the reaction vessel containing 20 mg (51 μmol) Kryptofix 2.2.2 and 20 μL of a 1*N* K_2CO_3 solution dissolved in 2 mL dry CH_3CN. The water was removed by evaporation and azeotropic distillation at 100°C using a stream of helium gas. To the anhydrous residue was added a solution of 2 to 3 mg (2.1–3.2 μmol) N^2-(p-anisyldiphenylmethyl)-9-[(4-tosyl)-3-p-anisyldiphenylmethoxy-methylbutyl]-guanine[39,40] in 0.6 mL dry dimethylformamide (DMF). The stirred reaction mixture was heated for 15 minutes at 120°C. The following acidic hydrolysis of the protection groups was performed by addition of 0.25 mL 1*N* HCl at 110°C for 5 minutes. The cooled reaction mixture was diluted with 1.5 mL of a 0.5*N* NaOAc solution and passed through a Alumina N Sep-Pak cartridge (Waters GmbH, Eschborn, Germany) activated previously by flushing with 10 mL EtOH followed by 30 mL deionized water to remove unreacted [^{18}F]fluoride. The crude product was injected into a semipreparative high-performance liquid chromatographic (HPLC) column (CS Chromatographie Service GmbH, Langerwehe, Germany; LiChrosorb 60 RP Select B-5μ; 250 × 10 mm; 5 μm; mobile phase: 0.9% aqueous NaCl:EtOH = 93:7; flow rate: 4.5 mL/min; 254 nm ultraviolet and radioactivity detector). Pure [^{18}F]FHBG, eluted off the column with a retention time of 7 to 8 minutes, was passed through a 0.2 μm Sterifix membrane filter (Braun-Melsungen AG, Melsungen, Germany) into a sterile multidose vial. Radiochemical yield was determined by analytic HPLC (CS Chromatographie Service; LiChrosorb 60 RP Select B-5μ; 250 × 4.6 mm; 5 μm; mobile phase: H_2O:EtOH = 90:10; flow rate: 1.0 mL/min; 254 nm ultraviolet and radioactivity detector) and was found to be greater than 97%. Under analytic conditions, the retention time for [^{18}F]FHBG was 9 minutes. The average radiochemical yield in 10 production runs was 7.2 ± 3.3% (end of synthesis [EOS], corrected for decay), and the synthesis time was 65 to 75 minutes from the end of bombardment. The specific activity was estimated to be > 3 GBq/μmol. The final product was tested for sterility and pyrogenicity by standard techniques.

Moreover, radiosynthesis of [^{18}F]2-fluoro-2-deoxy-D-glucose ([^{18}F]FDG) and [^{11}C]racloprid has been performed as described previously.[41,42]

Multimodal MRI and MicroPET

T_1-weighted MRIs for localization of tumors were obtained in experimental animals ($N = 12$ nude mice) on a 1.5 T Philips Gyroscan Intera prior to in vivo transduction. Multitracer microPET of experimental animals was performed as described previously.[21,43,44] No-carrier-added [^{18}F]FDG, [^{18}F]FHBG, or [^{11}C]racloprid was administered intravenously via the tail vein into experimental animals with doses ranging from 100 to 300 μCi/mouse for [^{18}F]FDG and [^{18}F]FHBG or 0.8 to 1.0 mCi/mouse for [^{11}C]racloprid. To study tracer accumulation and washout, PET was performed using a microPET (Concorde Microsystems Inc., Knoxville, TN; 63 image planes; 2.0 mm full width at half maximum[45]). Multiple emission scans (duration 10–50 minutes) were obtained starting at 30 minutes until 5 hours after tracer application. Images were reconstructed using Fourier rebinning (FORE) and two-dimensional filtered back projection without correction for attenuation and scatter. For quantification of images, a set of corresponding tracer reference standards of different radioactivity concentrations was placed within the field of view of the PET scanner. To allow image coregistration, a newly developed software was used allowing for fast automated coregistration of multimodal imaging data as described previously (*VINCI*, Vollmar, Cologne, Germany).[46] Data evaluation was based on a ROI analysis of PET images to determine maximal radioactivity concentrations within tumors. After background (mediastinum) subtraction, data were decay corrected and divided by the total injected dose to represent percentage injected dose per gram (%ID/g). As vectors have to be applied into viable tumor tissue to ensure expression of target genes, a protocol for acquisition of a series of multimodal images was established that allows identification of viable tumor tissue before application of vector particles using [^{18}F]FDG-PET. FDG-PET images were coregistered to MRIs and used for targeted vector application. Following the injection of HSV-DITG amplicon vectors into viable tumor, vector-mediated gene expression in vivo was analyzed by the use of the *d2r80A* as a PET marker gene and the specific receptor binding compound [^{11}C]racloprid and the *tk39gfp* as a PET marker gene and its specific substrate [^{18}F]FHBG. [^{11}C]RAC- and [^{18}F]FHBG-PET images acquired early after tracer injection were used for coregistration with the other imaging modalities. Repeat imaging later than 120 minutes after radiotracer injection was used to evaluate specific [^{11}C]RAC binding and [^{18}F]FHBG accumulation in transduced tumor portions to ensure quantification of the "tissue dose" of vector-mediated gene expression (in %ID/g).

Histology and Immunohistochemistry

Image validation has been performed as described previously.[21,47]

Statistics

Mean ± SD of the different radioactivity concentrations within the tumors and mediastinum of animals were calculated by Microsoft *Excel* 2000 (Microsoft Corp., Redmond, WA). Analysis of variance was performed by Superior Performance Software *SPSS* version 10.0 for Windows (SPSS Inc., UK Ltd, Surrey, England), and for regression analysis, *KaleidaGraph* 3.08 (Synergy Software, Reading, PA) was used.

Results

Construction of HSV-1 Amplicon Vector C-expressing D2R80A and TK39GFP

A mutated rat *d2r* (D80A) coding sequence was used to construct a HSV-1 amplicon vector to be used for noninvasive imaging of D2R80A expression. The D80A mutation leads to uncoupling of ligand binding from downstream signaling events. After amplification by PCR, the sequence was inserted into an IRES-based expression vector carrying the *tk39gfp* sequences.[21] In the resulting pHSV-*d2r80A*-IRES-*tk39gfp* (pHSV-DITG) vector construct, the *d2r80A* and *tk39gfp* genes are transcriptionally linked under the control of the immediate early promoter of the CMV (see Figure 1A). The *tk39* gene is fused to the *egfp* gene by a penta-glycine linker.[21] The *d2r80A* and the *tk39gfp* genes were joined by an IRES element leading to the production of a single *d2r80A-IRES-tk39gfp* transcript but uncoupled translation, thereby resulting in two distinct proteins (D2R80A and TK39EGFP).

Analysis of Appropriate Expression and Localization of D2R80A

A necessary prerequisite to use D2R80A in PET experiments is its expression and appropriate localization into the plasma membrane. To check the expression of the mutated D2 receptor, human Gli36dEGFR glioma cells were infected with HSV-DITG using MOI of 1 and 3 for analysis by SDS-PAGE and Western blotting. As indicated in Figure 1B, infected Gli36dEGFR cells expressed MOI-dependent increasing levels of D2R80A compared with untreated Gli36dEGFR cells and brain homogenates serving as negative and positive controls, respectively.

To further analyze the appropriate localization of D2R80A into the plasma membrane, Gli36dEGFR cells were infected with HSV-DITG and stained for D2R80A. TK39EGFP expression was located to the nucleus and cytoplasm, whereas D2R80A expression was mainly located in the plasma membrane (Figure 1C). As translation from HSV-DITG is uncoupled, two independent proteins emerged from the messenger ribonucleic acid, which explains the different localization of D2R80A and TK39GFP (see Figure 1C).

Quantitative Coexpression of D2R80A and TK39GFP as Assessed by PET

To investigate the sensitivity of PET to distinguish various levels of *d2r80A* and *tk39gfp* gene expression and to correlate the expression intensity of both genes in vivo, human Gli36dEGFR glioma cells were stably transfected with HSV-DITG, selected, and fluorescence-activated cell–sorted to generate distinct populations of transduced Gli36dEGFR-DITG cells with either low (R2) or high (R5) levels of GFP expression. For microPET imaging experiments, Gli36dEGFR-DITG-R2 ($n = 6$) and Gli36dEGFR-DITG-R5 ($n = 6$) cells were used as positive control tumors. As indicated in Figure 2, MRI and multitracer (RAC, FHBG) microPET images were acquired 24 hours after targeted vector administration into viable tumor tissue as depicted by FDG-PET to image *tk39gfp* gene expression and to compare it with *d2r80A* gene expression. A full set of wild-type and positive control tumors grew in 8 of 12 experimental animals as depicted by MRI. Therefore, results are based on the evaluation of eight experimental animals. A high rate of glucose metabolism was observed in all tumors. Specific RAC binding and FHBG retention were observed in seven of eight animals. RAC binding and FHBG retention were substantially greater in the HSV-DITG-transduced tumor (left flank) and in the positive control tumor (neck) than in the nontransduced negative control tumor (see Figure 2). The average racloprid binding was 1.1 ± 0.48%ID/g (in vivo transduced tumor) and 1.3 ± 0.6%ID/g (positive control tumors) and higher than in negative control tumors (0.9 ± 0.36%ID/g). The average FHBG retention was 0.5 ± 0.29%ID/g (in vivo transduced tumor) and 1.1 ± 1.08%ID/g (positive control tumors) and substantially higher than in negative control tumors (0.28 ± 0.17%ID/g). Racloprid binding to the endogenous D2 receptor in the striatum was quantified as an additional internal positive control and was found to be in the range of in vivo transduced and positive control tumors (1.6 ± 0.3%ID/g). In contrast, no specific FHBG accumulation could be observed within the brain (0.0059%ID/g). Most importantly, the quantitative assays for receptor binding (RAC-PET) and enzymatic assay (FHBG-PET) correlated to each other. Subcutaneous growing human Gli36dEGFR-DITG-R2 and Gli36dEGFR-DITG-R5 tumors were investigated with regard to racloprid binding and FHBG retention (%ID/g). The specific binding of racloprid (% ID/g) was plotted against the specific accumulation of FHBG (%ID/g). Regression analysis demonstrated a good correlation between the levels of functional *d2r80A* and *tk39gfp* gene activities ($r = .893$; Figure 3). Immunohistochemical analysis of in vivo HSV-1 amplicon–injected tumors supported the finding that D2R80A expression was mainly located in the plasma membrane (Figure 4, A–C), whereas TK39EGFP expression was located to the nucleus and cytoplasm (Figure 4, D–F). Taken together, these results suggest that *d2r80A* is an effective PET reporter gene that has the potential advantage of a blood-brain barrier–permeable ligand.

Discussion

The purpose of the present study was to further characterize a mutated D2R as a reporter gene for noninvasive imaging of gene expression mediated by a universal HSV-1 amplicon vector in a glioma model in nude mice. The generated bicistronic HSV-DITG vector mediates functional coexpression of *d2r80A* and HSV-1-*tk* on a quantitative level. Our data indicate that quantitative imaging data obtained by the receptor binding assay (*d2r80A*) and the enzymatic assay (*tk39gfp*) correlate to each other, at least for the levels of gene expression as obtained after in vivo transduction by HSV-1 amplicon vectors. Moreover, *d2r80A* in conjunction with its lipophilic binding compounds might be a good candidate as a marker gene for application of gene expression studies in the brain.

For safe and efficient gene therapy in clinical applications, technologies are required that allow noninvasive monitoring of the level and distribution of vector-mediated gene expression in vivo.[11] Three indirect radionuclide-based reporter gene approaches have been used for molecular PET: (1) enzyme-based reporter genes whose products convert radionuclide-labeled substrates to trapped products (eg, HSV-1-*tk*), (2) receptor-based reporter genes whose products bind radionuclide-labeled ligands (eg, *d2r* or *sstr2*), and (3) membrane transporter–based reporter genes (eg, the human sodium iodide symporter *hnis*[48]). Interest in the HSV-1-*tk* gene as an enzyme-based PET reporter gene has partly derived from the extensive investigation of HSV-1-*tk* as a therapeutic

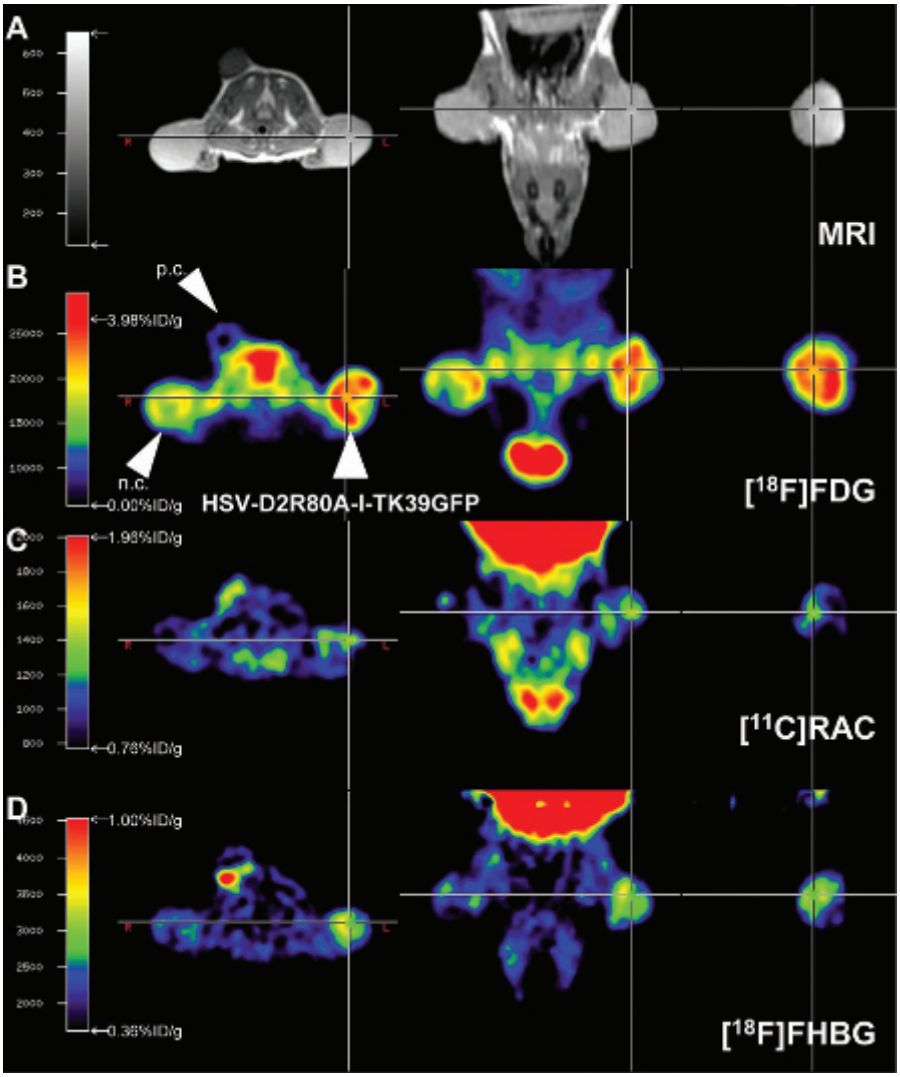

Figure 2. In vivo coregistration of HSV-DITG-mediated *d2r80A* and *tk39gfp* expression in nude mice bearing subcutaneous Gli36dEGFR tumors. Each animal had two subcutaneous Gli36dEGFR wild-type tumors on both upper flanks and one Gli36dEGFR-DITG tumor in the neck, which served as a positive control (p.c.). Magnetic resonance imaging (MRI) and [^{18}F]2-fluoro-2-deoxy-D-glucose positron emission tomomgraphy (FDG-PET) were performed prior to vector application. HSV-1 amplicon vector (HSV-DITG) was injected at doses ranging from 1.8×10^7 to 2.4×10^8 tu directly into the left Gli36dEGFR wild-type tumor along a craniocaudal axis of the animal 24 hours prior to further PET imaging. The right Gli36dEGFR wild-type tumor was left untreated and served as a negative control (n.c.). [^{11}C]Racloprid (RAC) and 9-(4-[^{18}F]fluoro-3-hydroxymethylbutyl)guanine (FHBG)-PET was performed 24 to 48 hours after vector application. *A*, MRI obtained at 1.5 T for exact tumor localization. *B*, [^{18}F]FDG-PET demonstrating viable and potentially proliferation tumor regions. Tumors that showed signs of necrosis were excluded from vector administration. *C*, RAC-PET demonstrating regions of D2R80A-related radioactivity primarily around injection sites, p.c. tumor and in the striatum. *D*, [^{18}F]FHBG-PET revealing regions of *tk39gfp*-related radioactivity primarily around injection sites and the p.c. tumor with no FHBG accumulation in the brain. The specific radioactivity concentration was assessed from PET images after subtraction of background activity (mediastinum).

gene for cancer treatment. HSV-1-TK can phosphorylate the acycloguanosines acyclovir, ganciclovir, and penciclovir. When administered at pharmacologic doses, HSV-1-TK-dependent production of the phosphorylated products of the acycloguanosines can cause death of cells expressing HSV-1-TK both by inhibiting the activity of DNA polymerase and by acting as a chain-terminating substrate.[49] Cells expressing HSV-1-TK can also kill neighboring cells via the "bystander effect." Consequently, a number of clinical trials in which HSV-1-TK expression in cancer cells is used to convert the acycloguanosine prodrugs into toxic agents have either been completed or are ongoing.[4] Furthermore, HSV-1-TK-dependent accumulation of 2′-fluoro-2′-deoxy-1β-D-arabinofuranosyl-5-[^{124}I]iodo-uracil ([^{124}I]FIAU) has been used following administration of a liposomal delivery vehicle containing

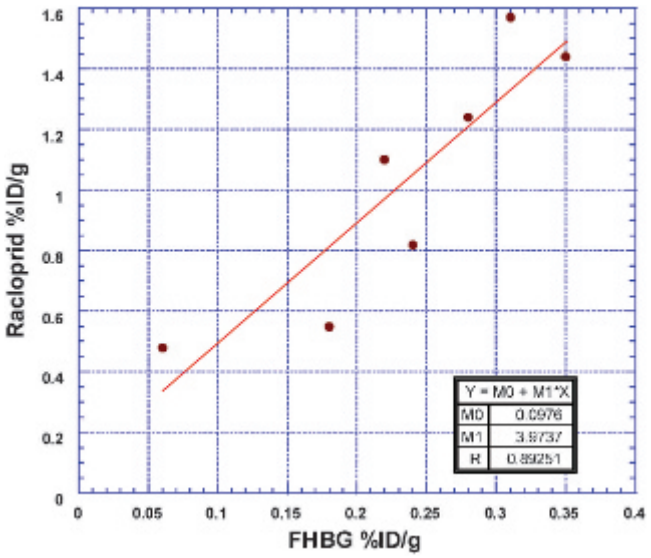

Figure 3. Correlation of *d2r80A* and *tk39gfp* expression. [^{11}C]Racloprid and 9-(4-[^{18}F]fluoro-3-hydroxymethylbutyl)guanine ([^{18}F]FHBG) positron emission tomographic data obtained from stably expressing Gli36dEGFR-DITG-R2 and Gli36dEGFR-DITG-R5 cells showing a good correlation between specific [^{11}C]racloprid binding and [^{18}F]FHBG-accumulation (r = .893).

an HSV-1-*tk* expression plasmid to image transduction efficiency in a clinical application by PET.[11] PET allows the quantitative localization of expression of endogenous or exogenous genes that code for enzymes or receptors by measuring the accumulation rate of enzyme substrates or the specific binding of receptor binding compounds in vivo.[50] The unique ability of the HSV-1-TK protein to selectively phosphorylate radiolabeled nucleoside analogues leading to their accumulation within the cells[10,12,51] allows the noninvasive imaging of *tk* gene expression in distinct regions within a transduced tissue. Most importantly, various levels of TK expression can be distinguished noninvasively by PET.[2,48]

As some therapeutic genes do not have an enzymatic or receptor function or no suitable substrate for detection by PET, HSV-1-*tk* can be used as a PET marker gene for the indirect quantification of a second linked and proportionally coexpressed gene. To enable the imaging of any gene of interest, multiple gene constructs have been generated serving a proportional coexpression of imaging and target genes. Approaches include the generation of fusion genes[36,52] and constructs serving IRES-based[53,54] or coordinately promoter-based[20] coexpression of the HSV-1-*tk* as a PET marker gene and a second gene of interest. Tjuvajev and colleagues, using xenografted tumors expressing varying levels of a bicistronic message containing both the HSV-1-*tk* gene and the *lacZ* gene, demonstrated a correlation between the in vivo measurement of the HSV-1-TK reporter activity with radiolabeled FIAU detected by single-photon emission computed tomography (SPECT) and β-galactosidase activity in tumor extracts.[53] In this experiment, the host animals had to be killed to measure β-galactosidase activity. Consequently, only a single correlation measurement could be obtained for each tumor sample.

It should be pointed out that HSV-1-*tk* is a foreign gene and may therefore raise an immune response. Reporter gene immunogenicity may lead to cell killing or reduced reporter gene expression over time. Moreover, current HSV-1-TK substrates cannot pass the blood-brain barrier.[11,23] Therefore, HSV-1-*tk* cannot be used to image reporter gene expression in the brain, unless some method of permeabilization of the blood-brain barrier is employed

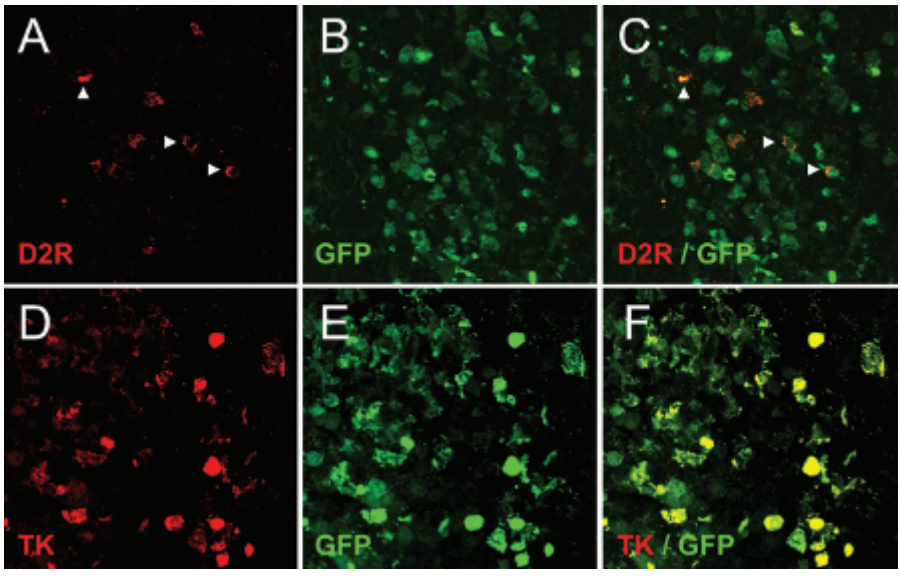

Figure 4. Localization of the mutated D2 receptor (D2R) in vivo. Sections of in vivo transduced Gli36dEGFR tumors by HSV-DITG were processed for immunohistochemical analysis. Immunostaining using antibody sc-9113 recognizing the N-terminal region of the D2R (*A–C*) and antibody against TK39 (*D–F*) was performed. *A*, D2R; *B*, green fluorescent protein (GFP); *C*, merge; *D*, TK39; *B*, GFP; *C*, merge. *Arrows* indicate the localization of the D2R in the plasma membrane. TK = thymidine kinase.

or unless it is used in a situation in which the blood-brain barrier is compromised (eg, brain tumors[11]). To solve some of these limitations, humanized (mitochondrial) versions of TK are being explored (J. Gelovani et al, personal communication, 2005), lipophilic HSV-1-TK marker substrates are being developed,[25] and alternative marker gene/marker substrate combinations are being characterized.[26,29]

One of the alternative marker gene/marker substrate combinations includes the use of the D2R, which specifically binds various carbon 11– or fluorine 18–labeled compounds, which are already being used in human application for the characterization of patients with Parkinson's disease.[55] Some of these compounds have been used in experimental application to image the expression of transduced wild-type[29] or mutated[34] D2R expression employing compounds such as 3-(2′-[^{18}F]-fluoroethyl)spiperone ([^{18}F]FESP),[29,34,54,56,57] [^{11}C]racloprid, [^{11}C]nemonapride, [^{11}C]N-methylspiperone,[58] and 11C-FLB457.[59] The most extensive investigations come from Gambhir and colleagues with applications of the wild-type and mutated D2R in combination with [^{18}F]FESP for imaging adenoviral vector–mediated gene delivery to the liver[29,34,57] and heart.[56] Since *d2r* is an endogenous gene, its use as a PET reporter gene has the advantage that its expression should not evoke an immune response. Moreover, its ligand [^{11}C]racloprid, which we used in this study as a reporter probe, is highly hydrophobic, allowing it to rapidly penetrate most membranes, including the blood-brain barrier.[60] The positive correlation between [^{11}C]racloprid binding and [^{18}F]FHBG accumulation, found in this study in gliomas and demonstrated by Yu and colleagues in stably expressing tumor clones, by Chen and colleagues in the heart, and by Liang and colleagues in the liver, employing [^{18}F]FESP instead of [^{11}C]racloprid,[54,56,57], points out that, at least for the levels of gene expression investigated, the receptor binding assay gives comparable quantitative information on transduced gene expression as the enzymatic assay using HSV-1-TK. However, it should be kept in mind that on a theoretical basis, the enzymatic assay is nonsaturable, enabling imaging of high levels of enzyme expression in contrast to a receptor binding assay, which is limited by the number of receptors being expressed on the cell surface.

In addition to the *d2r* and HSV-1-*tk* reporter genes, the *sstr2* receptor gene has also been used as a reporter for in vivo imaging with radioactive tracers. In this case, the ectopically expressed SSTr2 receptor can be imaged with [^{111}In]-, [^{188}RE]-, and [^{99m}Tc]-labeled ligands using SPECT.[27,61] Hemminki and colleagues described an adenovirus expressing both the HSV-1-*tk* and *sstr2* coding regions from a dual-reporter system in which each gene is expressed from a separate CMV promoter.[62] In this system, no correlation between imaging findings with regard to location and magnitude of the expression of the two reporter genes has been found in vivo.

Although signal amplification is a theoretical advantage of an enzyme-linked assay, the membrane permeability of the D2R ligand racloprid is a significant benefit, especially for imaging and treatment of brain tumors. And although D2R has been used as a PET reporter gene before as described above, it has never been imaged after HSV-1 amplicon vector–mediated transduction in vivo in a glioma model. Because of the benefits of D2R as a potential PET reporter gene, we generated and functionally analyzed an HSV-1 amplicon vector bearing transcriptionally linked genes of interest that serve proportional coexpression of three gene functions: (1) a marker gene for HSV-1 vector generation in cell culture (*gfp*); (2) a known and well-characterized PET marker gene that allows the evaluation of HSV-1 vector–mediated gene expression in vivo (*tk39*); (3) and a second reporter gene (*d2r80A*) to be characterized as an in vivo PET marker gene in this study. To avoid unwanted cellular responses owing to expression of *d2r80A*, we used a receptor bearing a mutation that uncouples ligand binding from downstream signaling events as described by Liang and colleagues.[34] We observed high expression of D2R80A and TK39GFP after infection of human Gli36dEGFR glioma cells with HSV-DITG, and D2R80A was correctly integrated into the plasma membrane (see Figures 2 and 3). Functional proportional coexpression of the PET marker genes *d2r80A* and *tk39gfp* could be imaged by [^{11}C]racloprid- and [^{18}F]FHBG-PET (see Figures 2 and 3).

One major disadvantage of using DNA delivery vehicles expressing bicistronic transcription units is the attenuation of protein expression that appears to be almost universal for the coding region placed distal to the IRES. In addition to reducing sensitivity for expression of the distal coding unit, the degree of attenuation may vary from tissue to tissue and from tumor to tumor.[63] As a result, the correlation between reporter gene expression and therapeutic gene expression may differ from one tissue or tumor to the next. However, HSV-1 amplicon–mediated *tk39gfp* gene expression could be imaged in vivo even with the *tk39gfp* gene located at the weak position downstream of IRES. These results present a significant step toward noninvasive imaging of gliomas in the clinical application with the major benefit of racloprid's ability to pass the blood-brain barrier. Therefore, these results may be useful

for the development of efficient and safe gene therapy protocols for brain tumors.

In conclusion, the *d2r80A* is an effective PET reporter gene with the advantage of a blood-brain barrier–permeable ligand. HSV-1 amplicon vector–mediated coexpression of *d2r80A* and *tk39gfp* demonstrates that receptor- and enzyme-based imaging approaches reveal similar results on a quantitative level for the levels of gene expression mediated by HSV-1 amplicon vectors in this study.

Acknowledgments

We thank G. Schneider and C. Selbach for their excellent technical assistance.

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